Cleavage Specificity on Synthetic Peptide Substrates of Human Rhinovirus 2 Proteinase 2A*

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Proteinase 2A of human rhinovirus serotype 2 (HRV2 2A) was expressed in E. coli and partially purified; the preparation was used to study various enzymatic parameters. Using a 16-amino acid peptide representing the native cleavage region of HRV2 2A, an apparent $K_{m}$ value of $5.4 	imes 10^{-4}$ mol/liter was determined. A minimum of 9 amino acids (comprising residues P8 to P1') was necessary for cleavage to occur. Proteolysis of substituted peptides was highly tolerant toward changes at P1, P2', and P3' but an absolute requirement for glycine P1' and a high preference for threonine P2 was found. Furthermore, HRV2 2A only cleaved peptide substrates derived from other rhinovirus serotypes and poliovirus that possessed P2 Thr and P1' Gly. Thus, the sequence Thr-X-Gly may form the basis of the cellular cleavage site processed by rhinoviral 2As during viral replication. Studies with various inhibitors support the hypothesis that HRV2 2A belongs to a new class of cysteine proteinases.

A variety of eukaryotic viruses produce their own specific proteinases. Essential for the processing of viral protein precursors, they are sometimes also involved in proteolytic modifications of host cell proteins (Morrison, 1991). Picornaviruses are absolutely dependent on specific cleavages of their polyprotein during their life cycle (Krusselich et al., 1987; Hellen et al., 1989). This polyprotein is synthesized from a single open reading frame coding for about 2100 amino acids (Rueckert, 1990). In rhinoviruses (the main causative agent of the common cold), polioviruses, and coxsackieviruses, the first proteolytic event is carried out by the viral protein 2A, a proteinase which cleaves intramolecularly at its own NH2 terminus (Toyoda et al., 1986; Sommergruber et al., 1989). As the polyprotein is not normally observed in infected cells, this cleavage most probably occurs on the growing peptide chain (Toyoda et al., 1986). All but one of the remaining cleavages within the polyprotein are catalyzed by the second virus-encoded proteinase 3C or its precursor 3C′D, respectively (Hanecak et al., 1982; Ypma-Wong et al., 1988).

In addition to the intramolecular cleavage of the nascent polyprotein, the 2As of rhino-, polio-, and coxsackieviruses are also responsible for the cleavage of the p220 component of the cap-binding protein complex eIF-4F; this leads to inhibition of the translation of capped mRNAs and thus to a shut-off of host-cell protein synthesis (Krusselich et al., 1987; Lloyd et al., 1987; Jewell et al., 1990). Picornaviral RNAs are still translated, as they are not capped. Since a direct cleavage of p220 by 2A has not as yet been demonstrated, a mechanism has been proposed in which a cellular proteinase becomes activated by 2A during viral replication (Wimmer, 1989; Wyckoff et al., 1990). Neither the common target itself nor a canonical sequence cleaved by all 2As has been defined. 2A and 3C proteinases have a cysteine residue as the active site nucleophile flanked by sequences with a high similarity to those of serine proteinases (Argos et al., 1984; Gorbalenya et al., 1986; Sommergruber et al., 1989). Moreover, it has been proposed that 3C proteinases fold as trypsin-like serine proteinases and 2A proteinases as small bacterial serine proteinases (e.g., α-lytic proteinase from Lysobacter enzymogenes) despite limited sequence similarity away from the active site (Bazan and Fletterick, 1988; Gorbalenya et al., 1989). The relevance of this hypothesis will have to be determined by x-ray crystallography. In the absence of such data, putative catalytic triad and active site residues have been identified by site-directed mutagenesis (Ivanoff et al., 1986; Sommergruber et al., 1989; Yu and Lloyd, 1991).

This report describes the expression of human rhinovirus serotype 2 (HRV2) 2A proteinase 2A in bacteria and its partial purification. Enzymatic parameters, intermolecular substrate requirements, and the effect of proteinase inhibitors on HRV2 2A were determined.

MATERIALS AND METHODS

Synthesis of Oligonucleotides and Oligopeptides—Oligonucleotides were made on an Applied Biosystems DNA synthesizer; purification was performed on OPC cartridges as described by McBride et al. (1988). Peptide synthesis was by the solid-phase Merrifield technique (Merrifield, 1963) on peptide synthesizers from Milligen (model 9050) or Zinsser (model SMPS 350). Purification was by reversed-phase HPLC on a Hibar LiChrosorb RP18 column (250/25, Merck) using a linear gradient of H2O/trifluoroacetic acid (100:0.1) and acetonitrile/trifluoroacetic acid (100:0.1). Homogeneity was demonstrated by analytical HPLC under the same conditions using a Bakerbond wide pore C18 column. The correct amino acid composition of all peptides was verified by Plasma Desorption Mass Spectroscopy (on a Bio-ION BIN K20 instrument), amino acid analysis, and/or by NH2-terminal amino acid sequencing.

*This work was supported by the Fonds zur Foerderung der Wissenschaftlichen Forschung and Boehringer Ingelheim. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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General Methods—Restriction endonucleases and DNA modifying enzymes were obtained from New England Biolabs and Boehringer Mannheim and were used as recommended by the supplier. (4-Aminodinophenyl)methanesulfonyl fluoride and 3,4-dichloro-isocoumarin were obtained from Boehringer Mannheim; all other proteinase inhibitors were from Sigma. DNA manipulations were carried out using published protocols (Sambrook et al., 1989).

Construction of a Proharyotic Expression Vector for HRV2 Proteinase 2A—A BstEII/HindIII fragment of pEX2A (Sommergruber et al., 1989) encompassing all of the 2A coding region except the first 11 NH2-terminal amino acids was isolated and ligated to a double-stranded 16-mer oligonucleotide (5'-CATCGGCGCCGCGGATGACATGT-3') encoding an ATG start codon (underlined) followed by the first 11 NH2-terminal amino acids. The ATG codon is part of an Ncol recognition site which was then used for inserting the complete Met-2A coding region into an Ncol-HindIII-cleaved pPROK-1 expression vector (CLONTECH Laboratories). The resulting construction (pPROK-2A) allows the expression of the mature HRV2 2A proteinase upon induction with isopropyl thiogalactoside from the tac-promoter in Escherichia coli HB101. LB medium containing 100 mg/mL ampicillin was used as bacterial growth medium.

Peptide Cleavages—The peptide TRPITAGPSDMY1VH (P8-P8') was shown in earlier work to be cleaved by bacterial extracts containing HRV2 2A (Sommergruber et al., 1989); cleavage occurs between the Ala (COOH terminus of VP1) and Gly (NH2 terminus of 2A) residues. P8-P8' was added to 500 µL of HRV2 2A containing fractions in 50 mM Tris-HCl (pH 8.5), 450 mM NaCl, and 5 mM DTT to a concentration of 100 µM unless otherwise stated (final volume 525 µL) and the samples were incubated at 34 °C for 3 h. Aliquots (100 µL) were removed at 2, 4, 8, 16, and 32 min, the reaction was stopped, and the samples prepared for reverse-phase HPLC analysis as described (Sommergruber et al., 1989). Substrate and products were separated using either a short or a long HPLC gradient (see "Material and Methods") is indicated by filled circles. Positions and molecular weights of the marker proteins are indicated on the right; UV absorption was measured at 210 nm and 280 nm. Cleavage products were identified by comigration of reference peptides and/or by protein sequencing (Hunkapiller and Hood, 1983). The percentage of P8-P8' cleaved for each time point was determined by comparing the area of the COOH- and NH2-terminal product peaks with that of the remaining uncleaved peptide. For each substrate and its products, the area under their combined peaks was independent (<±5%) of the extent of conversion of the substrate.

For the determination of NaCl concentration and pH dependence, the cleavage of substrate cleaved after 32 min under the conditions given above was defined as 100% activity; values determined under other conditions were expressed relative to these.

For inhibition studies, the initial rate (product production/minute) was calculated in the presence of increasing concentrations of inhibitor; the IC50 is that concentration of inhibitor required to reduce this rate by 50%.

Cleavage Assays for Vmax/Km Determination—These were performed as described above except that a mixture of a standard peptide P8-P8' (as described under "Results and Discussion") and a test peptide, both at 100 µM, was used. Aliquots were removed at half-minute intervals up to 3 min to allow the initial rate of cleavage/minute to be determined. For inhibition studies, the initial rate (product production/minute) was calculated in the presence of increasing concentrations of inhibitor; the IC50 is that concentration of inhibitor required to reduce this rate by 50%.

RESULTS AND DISCUSSION

Expression and Purification of HRV2 2A Proteinase—To express the HRV2 2A proteinase in bacteria, a 10-ml culture (in the stationary phase) of HB101 bearing pPROK-2A was added to 90 ml of medium. After 2 h at 35 °C, the cultures were induced with 0.5 mM isopropyl thiogalactoside and incubated overnight. Cells were harvested by centrifugation (10 min, 4000 x g, 4 °C), and the pellets were resuspended in 36 ml of ice-cold 50 mM Tris-HCl (pH 8.5) and 5 mM DTT (buffer A). Cell disruption was achieved with an MSE ultrasonic power unit using three 20-s bursts at 0 °C. Cell debris was removed by low speed centrifugation (5 min, 5000 x g, 4 °C) in a Sorvall centrifuge, and the cell sap was subjected to high speed centrifugation in a Beckman Ti-50 rotor (30 min, 35,000 rpm, 4 °C). The supernatant was loaded onto a MONO Q 10/10 column which was then washed with 40 ml of buffer A followed by 40 ml of the same buffer containing 0.3 M NaCl; the proteinase was eluted with a linear gradient (80 ml) of 0.3-0.5 M NaCl in buffer A. Fractions containing active proteinase 2A were identified by the assay described under "Materials and Methods," with the main activity eluting at 450 mM NaCl (Fig. 1). No cleavage of P8-P8' was observed in control experiments with extracts containing the inactive 2A mutant Cys129-Ser (Sommergruber et al., 1989), excluding the presence of contaminating bacterial proteinases. Fractions containing 2A were pooled and used for the subsequent investigations.

Enzymatic Parameters of the HRV2 2A Proteinase—In order to establish optimal conditions for the determination of Km, the pH, temperature, and salt concentration optima of HRV2 2A were determined. The pH of pooled fractions 9-11 of the MONO Q column in 25 mM Tris-HCl (pH 8.5), 450 mM NaCl, and 5 mM DTT was adjusted by adding 0.1 volume of a 0.5 M solution of various buffers (Fig. 2). The pH optimum of HRV2 proteinase 2A was found to be between 7 and 8.5 (Fig. 2, filled circles). The stability of 2A was studied by preincubating the enzyme at different pH values for 20 min.
FIG. 2. Dependence of HRV2 2A proteinase activity on pH. The activity of HRV2 2A toward the peptide P8-P8’ was determined by HPLC analysis of cleavage products as described under "Materials and Methods." The dependence of the activity on pH (●—●) and the influence of a 20-min preincubation at various pH values followed by adjustment to about pH 8.5 (●—●) were determined. The buffers (final concentration 50 mM) were: A, sodium citrate/HCl; B, glycine/NaOH; C, glycine/HCl; D, phthalic acid/NaOH; E, Tris/HCl; F, CH, COOH/NaOH; G, NaH2PO4/NaHPO4.

FIG. 3. Lineweaver-Burk plot for the determination of $K_m$ value for HRV2 2A proteinase using the peptide P8-P8’. The results are an average of three separate experiments.

presence of an inhibitor in the E. coli extract cannot be excluded.

Classification of HRV2 2A Proteinase Using Group-specific Inhibitors—HRV2 2A was challenged with typical serine, cysteine, aspartic, and metalloproteinase inhibitors; the results are shown in Table II. When dimethyl sulfoxide, methanol, or ethanol were required for solubilization, they were also added to the respective controls. Furthermore, DTT was removed by dialysis against 50 mM Tris-HCl (pH 8.5) and 450 mM NaCl before addition of cysteine proteinase inhibitors. Specific aspartic proteinase inhibitors and typical metalloproteinase inhibitors were without effect except for EDTA at high concentrations. SH reactive agents such as iodoacetamide and N-ethylmaleimide strongly inhibited 2A whereas E-64 had no effect. Antipain (a peptide aldehyde inhibitor of Cys and Ser proteinases), chymostatin, and elastatin (peptide aldehyde inhibitors of chymotrypsin-like and elastase-like Ser proteinases, respectively) were very effective against 2A, whereas leupeptin (also a peptide aldehyde inhibitor of Cys and Ser proteinases) was not. Of the Ser proteinase inhibitors tosyl lysyl chloromethyl ketone and tosyl phenylalanine chloromethyl ketone, which also inhibit cysteine proteinases nonspecifically by alkylation of the SH group, only tosyl lysyl chloromethyl ketone was significantly active against HRV2 2A. Thus, inhibitor studies do not allow classification of HRV2 2A as a conventional serine or cysteine proteinase.

Peptide Length Requirement—The absolute length of peptide required for cleavage by HRV2 2A was examined as shown in Fig. 4; in general, initial rate values obtained at single saturating peptide concentrations (4 mM = $9 \times K_m$; this concentration was achieved by dissolving the peptide directly in the 2A containing fractions) correlate well with the determined ($V_{max}/K_m$)rel values.

The NH₂-terminal truncations show that peptide P7-P8’ was as efficiently cleaved as P8-P8’ (Fig. 4A), however, a 3-fold reduction in the extent of cleavage was observed with P6-P8’. P5-P8’ and P4-P8’ were reduced by 5- and 10-fold relative to P8-P8’, respectively, and P3-P8’ was no longer cleaved at all.

At the COOH terminus, in contrast, P8-P2’ was cleaved nearly as efficiently as P8-P8’ (Fig. 4B) and P8-P1’ was still cleaved, although to a 30-fold lower extent. However, in a...
similar experiment with peptides having only 6 NH₂-terminal amino acids, P6-P8’ was cleaved 2-fold more efficiently than P6-P7’, P6-P6’, and P6-P5’ (Fig. 4C). Neither further truncated substrates nor peptides lacking internal residues (e.g. P8-P8’ΔP1ΔP1’) were substrates for 2A. Likewise replacement of the P1 and P1’ residues with ε-amino caproic acid or statine also gave peptides which were not cleaved by 2A (data not shown).

In summary, the smallest cleavable asymmetric peptide is the 9-mer P8-P1’ and the smallest cleavable symmetrical peptide is the 12-mer P6-P6’. The extension of P6-P8’ to P8-P8’ (Fig. 4A) and of P6-P6’ to P8-P6’ (compare Fig. 4, B and C) show a 3- and 7-fold increase in \( V_{\text{max}}/K_{\text{m}} \) values, respectively; this underlines the importance of the outer residues in substrate recognition or in stabilization of the peptide structure, especially when the P’ region is truncated.

**Table II**

<table>
<thead>
<tr>
<th>Inhibitor class</th>
<th>Inhibitor</th>
<th>Highest concentration tested</th>
<th>( IC_{50} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metalloprotease</td>
<td>Epipamastatin</td>
<td>0.15 mmol/L</td>
<td>NI*</td>
</tr>
<tr>
<td></td>
<td>Foroxymathin</td>
<td>0.2 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Phosphoramidin</td>
<td>0.15 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Bestatin</td>
<td>0.1 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>Epipamastatin</td>
<td>0.35 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>EGTA</td>
<td>50 mmol/L</td>
<td>NI</td>
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<tr>
<td></td>
<td>EDTA</td>
<td>50 mmol/L</td>
<td>16.5</td>
</tr>
<tr>
<td></td>
<td>1,10-Phenanthroline</td>
<td>10 mmol/L</td>
<td>3.2</td>
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<tr>
<td>Asp protease</td>
<td>NLE-STA-ALA-STA</td>
<td>0.2 mmol/L</td>
<td>NI</td>
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<td></td>
<td>Pepstatin</td>
<td>0.35 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td>Ser protease</td>
<td>3,4-Dichloro-isocoumarin</td>
<td>2.1 mmol/L</td>
<td>1.45</td>
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<td></td>
<td>Elastatinal</td>
<td>0.5 mmol/L</td>
<td>0.055</td>
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<tr>
<td></td>
<td>APMSF</td>
<td>1.68 mmol/L</td>
<td>NI</td>
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<tr>
<td></td>
<td>Chymostatin</td>
<td>0.15 mmol/L</td>
<td>0.025</td>
</tr>
<tr>
<td>Cys protease</td>
<td>E-64</td>
<td>0.2 mmol/L</td>
<td>NI</td>
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<tr>
<td></td>
<td>Iodacetamide</td>
<td>1.25 mmol/L</td>
<td>0.08</td>
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<tr>
<td></td>
<td>NEM</td>
<td>0.7 mmol/L</td>
<td>0.03</td>
</tr>
<tr>
<td>Cys/Ser protease</td>
<td>Antipain</td>
<td>0.7 mmol/L</td>
<td>0.065</td>
</tr>
<tr>
<td></td>
<td>Leupeptin</td>
<td>0.45 mmol/L</td>
<td>NI</td>
</tr>
<tr>
<td></td>
<td>TPK</td>
<td>13 mmol/L</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>TLCK</td>
<td>0.1 mmol/L</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*IC₅₀ values represent the concentration of inhibitor required to reduce the enzyme activity by 50% determined as described under "Materials and Methods."

A NI, no inhibition found at the highest concentration tested.

Intramolecular Substrate Specificity on Amino Acid-substituted Peptide Substrates—To study the contribution of various amino acids to the substrate specificity of HRV2 2A, a set of 15-mer peptides bearing single amino acid substitutions was synthesized (Table III) and their cleavage compared to that of P8-P8’. Use of modified 15-mer (P8-P7’) peptides enabled a much better separation of substrate and products on HPLC; furthermore, there is no significant difference in the cleavage efficiency of wild-type P8-P8’ and P8-P7’ peptides (Fig. 4B). Table III shows the \( V_{\text{max}}/K_{\text{m}} \) values for peptides with substitutions at the indicated positions.

All changes at P2 (only substrates P2S, P2R, and P2N were processed to any significant extent) and P1’ (none of the peptides were processed) were highly deleterious. In contrast, all P2’- and P3’-substituted peptides were cleaved with \( V_{\text{max}}/K_{\text{m}} \) values reduced by between 1.5- to 5-fold. Of the 13 peptides with changes at P1, seven were poorly processed or not at all (P1V, P1P, P1N, P1Q, P1D, P1E, and P1K), and four (P1L, P1F, P1T, and P1R) were cleaved between 1.5 and 2.5 times less efficiently. However, replacement of the P1Ala with Met or Tyr resulted in 5.1- and 1.38-fold increases in \( V_{\text{max}}/K_{\text{m}} \) values, respectively. Interestingly, the enzyme was capable of distinguishing between Arg and Lys at P1; P1R was cleaved 4.5-fold more efficiently than P1K.

Taken together, these results suggest important roles for P2 and P1’ in enzyme-substrate interaction or in stabilizing peptide structure (or both) and a rather open binding site for the P1 position which can accommodate a variety of amino acids (Met, Leu, Tyr, Phe, Thr, and Arg) but not all (e.g. Val, Pro, Glu, and Asp). Substitution of P1Ala for Val (but not Leu or Met) also interfered with intramolecular HRV2 2A cleavage (Skern et al., 1991). In contrast, the substitutions P2N and P1Q only reduced intramolecular cleavage by about 10 and 20%, respectively, whereas both peptides are reduced 10-fold in \( V_{\text{max}}/K_{\text{m}} \) values. The bimolecular nature of trans cleavage may be responsible for this greater reduction in efficiency.

Efficiency of Cleavage of HRV2 2A on Peptides Derived from Other Rhino- and Polioviral 2A Cleavage Sites—The VP1/2A sequences of rhino-, polio-, and coxsackieviruses have been compared (Palmenberg, 1989; Skern et al., 1991, and references therein). Apart from the ubiquitous presence of glycine at P1’, there is considerable variation between and even among the genera. The efficiency of HRV2 2A cleavage on such peptides derived from other rhinovirus serotypes and from poliovirus type 1 (Mahoney) is shown in Table IV. Because of the lack of sequence information for the 2A regions

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*Effect of protease inhibitors on the activity of HRV2 2A protease*

The abbreviations used are: NLE-STA-ALA-STA, norleucine-statine-alanine-statine; TLCK, tosyl lysyl chloromethyl ketone; TPCK, tosyl phenylalanyl chloromethyl ketone.
of HRV1A, HRV1B, HRV39, and HRV49 chimeric peptides containing a P region corresponding to the COOH terminus of VP1 of the respective HRV serotype and the P' region of HRV2 were employed (Table IV).

The HRV85 and HRV49 peptides were cleaved 3-fold less efficiently by HRV2 2A than the cognate peptide; the presence of Ala at P6 in place of Pro might be responsible for the reduction, as the only other difference between HRV49 and HRV2 is the Thr to Ser change at P8. Four peptides were cleaved with efficiencies between 25- and 100-fold lower than that of P6-P8' (HRV1A, HRV39, PV1, and HRV1B) and three (HRV89, HRV14, and HRV9) were not cleaved at all. The processing of the poliovirus peptide (PV1) is surprising, as only 3 amino acids (P3, P2, and P1') are identical with the HRV2 peptide. However, as shown in Table III, P2 and P1' are extremely important in influencing substrate efficiency. It is thus possible that favored amino acids at these positions plus an acceptable P1 residue (for PV1, Tyr) represent a structure acceptable for HRV2 2A. Furthermore, none of the three serotype peptides with other amino acids at these positions were cleaved. These results are relevant to the second biological function of rhino- and polioviral 2As, namely the intermolecular reaction by which the polyprotein p220 is degraded and the translation of capped mRNAs by the host-cell is arrested. A common sequence must thus be recognized by all 2As; the inability of HRV2 2A to cleave efficiently the peptides in Table IV makes it difficult to propose a common target unless the configuration of P3 to P1' is favorable, as appears to be the case for the PV1 peptide.

In summary, a partial purification scheme for HRV2 2A proteinase has been developed and some enzymatic properties and the substrate specificity have been determined. Further work with HRV2 2A and other 2As will be required to elucidate
the target recognized in 2A-mediated induction of the host-cell shut off.

Acknowledgments—We thank C. Petric, M. Sluga, and A. Auinger for help as summer students; D. Jobstmann, T. Gramantisch, H. Hoffmann, E. Spielvogel, and G. Illibauer for excellent technical assistance; R. Hauptmann for supplying oligonucleotides and Z. Ratter for invaluably stimulating discussions.

Table IV

<table>
<thead>
<tr>
<th>Code*</th>
<th>Sequence*</th>
<th>Relative efficiency of cleavage (V_{mut}/K_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>P8-P8' (HRV2)</td>
<td>TRPI1TTA*GPSDMYVH</td>
<td>1.00</td>
</tr>
<tr>
<td>HRV49*</td>
<td>sRasITTAT*GPSDMYVH</td>
<td>0.30</td>
</tr>
<tr>
<td>HRV85</td>
<td>eRas1TTA*GPSDMYVH</td>
<td>0.31</td>
</tr>
<tr>
<td>HRV1A*</td>
<td>rRnt1TTA*GPSDMYVH</td>
<td>0.01</td>
</tr>
<tr>
<td>HRV39*</td>
<td>pRen1TTA*GPSDMYVH</td>
<td>0.04</td>
</tr>
<tr>
<td>PV1</td>
<td>stdl1TTY*Gfghqmnk</td>
<td>0.02</td>
</tr>
<tr>
<td>HRV14</td>
<td>rkgd1ksy*Glgrqygg</td>
<td>No cleavage</td>
</tr>
<tr>
<td>HRV1B*</td>
<td>pRasmkTv*GPSDMYVH</td>
<td>0.03</td>
</tr>
<tr>
<td>HRV9</td>
<td>nvravknv*GPSDMYVH</td>
<td>No cleavage</td>
</tr>
<tr>
<td>HRV89</td>
<td>dvftvTv*GPSAMYVH</td>
<td>No cleavage</td>
</tr>
</tbody>
</table>

* Indicates that the P1 region is that of HRV2.

Lowercase letters represent amino acids differing from those found in HRV2.

Competition cleavage reactions were carried out as described under "Materials and Methods"; efficiency of cleavage is expressed as (V_{mut}/K_{max}) using P8-P8' as reference peptide.

References

Cleavage specificity on synthetic peptide substrates of human rhinovirus 2 proteinase 2A.
W Sommergruber, H Ahorn, A Zöphel, I Maurer-Fogy, F Fessl, G Schnorrenberg, H D Liebig, D Blaas, E Kuechler and T Skern


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